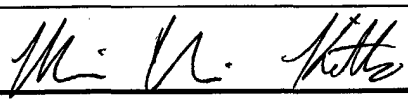
Applicants also contend that the present invention works with inhibitors other than inhibitors of metalloproteinase dependent cleavage. Schraufstatter et al., Oak et al., Filardo et al., Kalmes et al., McCole et al., Yan et al., and Pai et al. (abstracts attached) clearly demonstrate that other types of inhibitors are capable of blocking receptor tyrosine kinase transactivation. Also attached is experimental data from one of the present inventors showing that anti-HB-EGF antibodies are capable of blocking receptor tyrosine kinase transactivation. If necessary, this data can be submitted in the form of a declaration. Applicants also point out that the experiments in the present application show that the EGFR kinase antagonist AG1478 is capable of blocking transactivation of an EGFR/PDGFR chimeric receptor.

In addition, applicants point out that contacting the cell with a compound capable of activating the G-protein mediated signal transduction pathway is not necessary in the present invention. Plonowski et al., Prenzel et al., Lin and Freemen, Guo et al, Gallet et al., Wan et al., Asakura et al., and Buteau et al., demonstrate that transactivation of receptor tyrosine kinases also takes place in other types of cells, e.g. heart cells, cultured fibroblasts or pancreatic beta-cells. Figure 4(g) in the present application shows that a high constitutive phosphotyrosine content of EGFR in unstarved PC3 cells (without contacting the cell with a stimulating compound) is reduced by treatment with BB-94 (page 16, lines 13-15).

In view of the attached references, applicants respectfully contend that the present claims are enabled and submit that all of claims 22-36 are in condition for allowance. If it is believed that the application is not in condition for allowance, it is respectfully requested that the undersigned attorney be contacted at the telephone number below.

In the event this paper is not considered to be timely filed, the Applicant respectfully petitions for an appropriate extension of time. Any fee for such an extension together with any

additional fees that may be due with respect to this paper, may be charged to Counsel's Deposit Account No. 02-2135.

RESPECTFULLY SUBMITTED,					
NAME AND REG. NUMBER	Monica Chin Kitts, Registration No.: 36,105				
SIGNATURE			*DATE*	Feb. 21, 2003	
ADDRESS	Rothwell, Figg, Ernst & Manbeck Suite 800, 1425 K Street, N.W.				
CITY	Washington	*STATE*	D.C.	*ZIP CODE*	20005
COUNTRY	U.S.A.	*TELEPHONE*	202-783-6040	*FAX*	202-783-6031

APPENDIX

Marked-Up copy of claims to show amendments:

36.(Amended) A method for modulating a G-protein mediated signal transduction comprising:

providing a cell having a disturbed G-protein mediated signal transduction and a receptor tyrosine kinase capable of transactivation by G-protein mediated signal transduction, wherein said receptor tyrosine kinase is selected from the group consisting of EGFR and other members of the EGFR family, said ~~cell~~ receptor tyrosine kinase comprising an extracellular domain and said cell having a G-protein mediated signal transduction pathway wherein one or more tyrosine residues are phosphorylated based on the activation of signal transduction pathway, the extracellular domain of said receptor is capable of binding to its receptor ligand, and said ligand is generated from a precursor of said ligand by a proteinase-dependent cleavage;

contacting said cell with a compound affecting an extracellular G protein or G protein coupled receptor initiated signal pathway resulting in the activation of the receptor tyrosine kinase and thereby modulating the receptor tyrosine kinase activation by G-protein mediated signal transduction.

Relevant references relating to the point that the process of the present invention cannot only be used for EGFR but also for other receptor tyrosine kinases:

Transactivation of the PDGFR

Neuron 2002 Sep 12;35(6):1111-22

A D2 class dopamine receptor transactivates a receptor tyrosine kinase to inhibit NMDA receptor transmission.

Kotecha SA, Oak JN, Jackson MF, Perez Y, Orser BA, Van Tol HH, MacDonald JF.
Department of Physiology, Faculty of Medicine, University of Toronto, Ontario, Canada.

Receptor tyrosine kinases (RTKs) are membrane spanning proteins with intrinsic kinase activity. Although these receptors are known to be involved in proliferation and differentiation of cells, their roles in regulating central synaptic transmission are largely unknown. In CA1 pyramidal neurons, activation of D2 class dopamine receptors depressed excitatory transmission mediated by the NMDA subtype of glutamate receptor. This depression resulted from the quinpirole-induced release of intracellular Ca(2+) and enhanced Ca(2+)-dependent inactivation of NMDA receptors. The dopamine receptor-mediated depression was dependent on the "transactivation" of PDGFRbeta. Therefore, RTK transactivation provides a novel mechanism of communication between dopaminergic and glutamatergic systems and might help to explain how reciprocal changes in these systems could be linked to the deficits in cognition, memory,and attention observed in schizophrenia and attention deficit hyperactivity disorder

J Biol Chem 2002 Dec 11; [epub ahead of print]

Sphingosine 1-phosphate and platelet-derived growth factor act via platelet-derived growth factorbeta receptor-sphingosine 1-phosphate receptor complexes in airway smooth muscle cells.

Waters C, Sambi BS, Kong KC, Thompson D, Pitson SM, Pyne S, Pyne NJ.
Physiology and Pharmacology, University of Strathclyde, Glasgow, Scotland G4 0NR.

Platelet-derived growth factor and sphingosine 1-phosphate (S1P) act via PDGFbeta receptor-S1P1 receptor complexes in airway smooth muscle cells to promote mitogenic signaling. Several lines of evidence support this conclusion. First, both receptors were co-immunoprecipitated from cell lysates with specific anti-S1P1 antibodies, indicating that they form a complex. Second, treatment of ASM cells with PDGF stimulated the phosphorylation of p42/p44 MAPK and this phosphorylated p42/p44 MAPK associates with the PDGFbeta receptor-S1P1 receptor complex. Third, treatment of cells with anti-sense S1P1 receptor plasmid construct reduced the PDGF- and S1P-dependent activation of p42/p44 MAPK. Fourth, S1P and/or PDGF induced the formation of endocytic vesicles containing both PDGFbeta receptors and S1P1 receptors, which was required for activation of the p42/p44 MAPK pathway. PDGF does not induce the release of S1P suggesting the absence of a sequential mechanism. However, sphingosine kinase1 (SK1) is constitutively exported from cells and supports activation of p42/p44 MAPK by exogenous sphingosine. Thus, the presentation of sphingosine from other cell types, and its conversion to S1P by the kinase exported from airway smooth muscle cells might enable S1P to act with PDGF on the PDGFbeta receptor-S1P1 receptor complex to induce biological responses in vivo. These data provide further evidence for a novel mechanism for GPCR and receptor tyrosine kinase signal integration that is distinct from the transactivation of receptor tyrosine kinases by GPCR agonists and/or sequential release

and action of S1P in response to PDGF.

Exp Cell Res 2002 Apr 1;274(2):264-74

Migration of vascular smooth muscle cells induced by sphingosine 1-phosphate and related lipids: potential role in the angiogenic response.

Boguslawski G, Grogg JR, Welch Z, Ciechanowicz S, Sliva D, Kovala AT, McGlynn P, Brindley DN, Rhoades RA, English D.
Experimental Cell Research Laboratory, Methodist Research Institute, Indianapolis, Indiana 46202, USA.

The bioactive lipids sphingosine 1-phosphate (SPP), sphingosylphosphorylcholine, and lysophosphatidic acid play an important role in angiogenesis as a result of their effects on both the migration of endothelial cells (ECs) and the integrity of EC monolayers. Here we show that extremely low concentrations of serum and nanomolar concentrations of these biologically active lipids stimulate migration of human aortic smooth muscle cells (SMCs). However, at dosages most effective in promoting EC migration and in enhancing EC monolayer integrity, serum and SPP potently inhibited SMC migration; SPP also blocked the migration induced by protein growth factors. Treatment of SMCs with SPP induced transient phosphorylation of a 175- to 185-kDa protein corresponding to the PDGF receptor, indicating transactivation of this receptor. SPP and related lipids may play a key role in angiogenesis by coordinating the migration of both endothelial cells and vascular smooth muscle cells in response to the changing gradients of these bioactive lipid messengers.

Mol Pharmacol 2001 Jul;60(1):92-103

Dopamine D(4) and D(2L) Receptor Stimulation of the Mitogen-Activated Protein Kinase Pathway Is Dependent on trans-Activation of the Platelet-Derived Growth Factor Receptor.

Oak JN, Lavine N, Van Tol HH
Laboratory of Molecular Neurobiology, Centre for Addiction and Mental Health, Toronto, Ontario, Canada.

The ability of dopamine D(4) and D(2) receptors to activate extracellular signal-regulated kinases (ERKs) 1 and 2 was compared using Chinese hamster ovary (CHO-K1) cells transfected with D(4.2), D(4.4), D(4.7), and D(2L) receptors. Dopamine stimulation of D(4) or D(2L) receptors produced a transient, dose-dependent increase in ERK1/2 activity. Receptor-specific activation of the ERK mitogen-activated protein kinase (MAPK) pathway was confirmed using the D(2)-like receptor-selective agonist quinpirole, whereas the specific antagonist haloperidol blocked activation. MAPK stimulation was dependent on a pertussis-toxin-sensitive G protein (G(i/o)). trans-Activation of the platelet-derived growth factor (PDGF) receptor was an essential step in D(4) and D(2L) receptor-induced MAPK activation. PDGF receptor-selective tyrosine kinase inhibitors tyrphostin A9 and AG1295 abolished or significantly inhibited ERK1/2 activation by D(4) and D(2L) receptors. Dopamine stimulation of the D(4) receptor also produced a rapid increase in tyrosine phosphorylation of the PDGF receptor-beta. The Src-family tyrosine kinase inhibitor PP2 blocked MAPK activation by dopamine; however, this drug was also found to inhibit PDGF-BB-stimulated ERK activity and autophosphorylation of the PDGF receptor-beta. Downstream signaling pathways support the involvement of a receptor tyrosine kinase. The phosphoinositide 3-kinase inhibitors wortmannin and LY294002, protein kinase C inhibitors GF109203X and

Calphostin C, dominant-negative RasN17, and the MEK inhibitor PD98059 significantly attenuated or abolished activation of MAPK by dopamine D(4) and D(2L) receptors. Our results indicate that D(4) and D(2L) receptors activate the ERK kinase cascade by first mobilizing signaling by the PDGF receptor, followed by the subsequent activation of ERK1/2 by pathways associated with this receptor tyrosine kinase.

J Mol Cell Cardiol 2001 Jan;33(1):3-7

Transactivation: a novel signaling pathway from angiotensin II to tyrosine kinase receptors.

Saito Y, Berk BC.
Center for Cardiovascular Research, University of Rochester, Rochester, NY, USA.

Angiotensin II (Ang II), an octapeptide pressor hormone, activates cellular events that may contribute to the pathogenesis of cardiovascular disease. The physiological actions of Ang II are mediated via the Ang II type 1 receptor (AT1R) and type 2 receptor (AT2R), which are G protein-coupled receptors (GPCR). GPCR share a common basic structure of seven transmembrane helices connected by alternating cytoplasmic and extracellular loops. GPCR lack intrinsic kinase activity possessed by receptor tyrosine kinases (RTK) such as platelet-derived growth factor receptor (PDGFR) or epidermal growth factor receptor (EGFR). Nonetheless, the signal transduction events activated by the AT1R mimic those of RTKs. Recently, cross-talk between GPCR and RTK has been observed. There is accumulating evidence that GPCR take advantage of signaling pathways downstream of RTK to exert its effect on the cells. In this context, RTK may be considered as one of signaling molecules downstream of GPCR

Mol Pharmacol 2000 Oct;58(4):814-20Related Articles, Links

The CB(1) cannabinoid receptor is coupled to the activation of c-Jun N-terminal kinase.

Rueda D, Galve-Roperh I, Haro A, Guzman M.
Department of Biochemistry and Molecular Biology I, School of Biology, Complutense University, Madrid, Spain.

Cannabinoids exert most of their effects through the CB(1) receptor. This G-protein-coupled receptor has been shown to be functionally coupled to inhibition of adenylyl cyclase, modulation of ion channels, and activation of extracellular signal-regulated kinase. Using Chinese hamster ovary cells stably transfected with the CB(1) receptor cDNA, we show here that Delta(9)-tetrahydrocannabinol (THC), the major active component of marijuana, induces the activation of c-Jun N-terminal kinase (JNK). Western blot analysis showed that both JNK-1 and JNK-2 were stimulated by THC. The effect of THC was also exerted by endogenous cannabinoids (anandamide and 2-arachidonoylglycerol) and synthetic cannabinoids (CP-55,940, HU-210, and methanandamide), and was prevented by the selective CB(1) antagonist SR141716. Pertussis toxin, wortmannin, and a Ras farnesyltransferase inhibitor peptide blocked, whereas mastoparan mimicked, the CB(1) receptor-evoked activation of JNK, supporting the involvement of a G(i)/G(o)-protein, phosphoinositide 3'-kinase and Ras. THC-induced JNK stimulation was prevented by tyrphostin AG1296, pointing to the implication of platelet-derived growth factor receptor transactivation, and was independent of ceramide generation. Experiments performed with several types of neural cells that endogenously express the CB(1) receptor suggested that long-term JNK activation may be involved in THC-induced cell death. The CB(1) cannabinoid receptor was

also shown to be coupled to the activation of p38 mitogen-activated protein kinase. Data indicate that activation of JNK and p38 mitogen-activated protein kinase may be responsible for some of the cellular responses elicited by the CB(1) cannabinoid receptor.

J Biol Chem 2000 May 26;275(21):15926-32

Angiotensin II induces transactivation of two different populations of the platelet-derived growth factor beta receptor. Key role for the p66 adaptor protein Shc.

Heeneman S, Haendeler J, Saito Y, Ishida M, Berk BC.

Center for Cardiovascular Research, University of Rochester, Rochester, New York 14642, USA.

Several signal transduction events induced by angiotensin II (AngII) binding to the angiotensin II type 1 receptor resemble those evoked by platelet-derived growth factor (PDGF) binding to the PDGF-beta receptor (PDGFbeta-R). We report here, in agreement with previous data, that AngII and PDGF-B-chain homodimer (PDGF-BB) stimulate tyrosine phosphorylation of the PDGFbeta-R. Both AngII and PDGF-BB stimulated the phosphorylation of PDGFbeta-R via the binding of tyrosine-phosphorylated Shc to PDGFbeta-R. Both PDGF-BB- and AngII-induced phosphorylation of the Shc.PDGFbeta-R complex was inhibited by antioxidants such as N-acetylcysteine and Tiron, but not by calcium chelation. However, transactivation of PDGFbeta-R by AngII (measured by PDGFbeta-R tyrosine phosphorylation) differed significantly from PDGF-BB. Evidence to support different mechanisms of PDGFbeta-R phosphorylation includes differences in the time course of PDGFbeta-R phosphorylation, differing effects of inhibitors of the endogenous PDGFbeta-R tyrosine kinase and Src family tyrosine kinases, differing results when the PDGFbeta-R was directly immunoprecipitated (PDGFbeta-R-antibody) versus coimmunoprecipitated (Shc-antibody), and cell fractionation studies that suggested that the Shc.PDGFbeta-R complexes phosphorylated by AngII and PDGF-BB were located in separate subcellular compartments. These studies are the first to suggest that transactivation of tyrosine kinase receptors by G protein-coupled receptors involves a unique pathway that regulates a population of tyrosine kinase receptors different from endogenous tyrosine kinase ligand.

Transactivation of the vascular endothelial growth factor receptor KDR/Flk-1

Nippon Yakurigaku Zasshi 2002 Nov;120(1):104P-105P

Transactivation of the vascular endothelial growth factor receptor KDR/Flk-1 by the bradykinin B2 receptor induces an angiogenic phenotype in human cultured coronary endothelial cells]

Miura S, Fujino M, Tanigawa H, Matsuo Y, Saku K.

Department of Cardiology, Fukuoka University School of Medicine, Fukuoka 814-0142, Japan.

BACKGROUND: Endothelial cells (ECs) are believed to be critical cellular elements responsible for postnatal angiogenesis. Vascular endothelial growth factor (VEGF) stimulates angiogenesis via the activation of KDR/Flk-1 receptor, which is mainly expressed in ECs. Transactivation of

KDR/Flk-1 receptor by bradykinin (BK) B2 receptor contributes to the activation of endothelial nitric-oxide (NO) synthase. Therefore, we examined whether transactivation by BK induced angiogenesis. METHODS AND RESULTS: We developed an in vitro model of human coronary artery ECs (HCECs) tube formation on a matrix gel. We demonstrated that BK dose-dependently induced tube formation. Although a lower concentration of BK did not induce tube formation, the combination of a lower concentration of BK and VEGF did. These effects blocked specific inhibitors of VEGF receptor tyrosine kinases (Tki) and NO synthase. In addition, BK induced the tyrosine phosphorylation of KDR/FlK-1 receptor (transactivation), as did VEGF itself. This transactivation was also blocked by Tki. CONCLUSIONS: Transactivation of KDR/Flk-1 by BK through B2 receptor is a potent signaling in angiogenic phenotype in HCECs.

J Biol Chem 2002 Nov 8;277(45):42997-3001

Transactivation of vascular endothelial growth factor (VEGF) receptor Flk-1/KDR is involved in sphingosine 1-phosphate-stimulated phosphorylation of Akt and endothelial nitric-oxide synthase (eNOS).

Tanimoto T, Jin ZG, Berk BC.

Center for Cardiovascular Research, University of Rochester, Rochester, New York 14642, USA.

Sphingosine 1-phosphate (S1P) and vascular endothelial growth factor (VEGF) elicit numerous biological responses including cell survival, growth, migration, and differentiation in endothelial cells mediated by the endothelial differentiation gene, a family of G-protein-coupled receptors, and fetal liver kinase-1/kinase-insert domain-containing receptor (Flk-1/KDR), one of VEGF receptors, respectively. Recently, it was reported that S1P or VEGF treatment of endothelial cells leads to phosphorylation at Ser-1179 in bovine endothelial nitric oxide synthase (eNOS), and this phosphorylation is critical for eNOS activation. S1P stimulation of eNOS phosphorylation was shown to involve G(i) protein, phosphoinositide 3-kinase, and Akt. VEGF also activates eNOS through Flk-1/KDR, phosphoinositide 3-kinase, and Akt, which suggested that S1P and VEGF may share upstream signaling mediators. We now report that S1P treatment of bovine aortic endothelial cells acutely increases the tyrosine phosphorylation of Flk-1/KDR, similar to VEGF treatment. S1P-mediated phosphorylation of Flk-1/KDR, Akt, and eNOS were all inhibited by VEGF receptor tyrosine kinase inhibitors and by antisense Flk-1/KDR oligonucleotides. Our study suggests that S1P activation of eNOS involves G(i), calcium, and Src family kinase-dependent transactivation of Flk-1/KDR. These data are the first to establish a critical role of Flk-1/KDR in S1P-stimulated eNOS phosphorylation and activation.

Transactivation of the Trk receptor

J Biol Chem 2002 Mar 15;277(11):9096-102

Activation of Trk neurotrophin receptor signaling by pituitary adenylate cyclase-activating polypeptides.

Lee FS, Rajagopal R, Kim AH, Chang PC, Chao MV.

Department of Psychiatry, Weill Medical College, Cornell University, New York, New York 10021, USA. fslee@med.cornell.edu

Pituitary adenylate cyclase-activating polypeptide (PACAP), a neuropeptide that acts through G protein-coupled receptors, exerts neuroprotective effects upon many neuronal populations. However, the intracellular signaling mechanisms that account for PACAP's trophic effects are not well characterized. Here we have tested the possibility that PACAP uses neurotrophin signaling pathways. We have found that PACAP treatment resulted in an increase in TrkA tyrosine kinase activity in PC12 cells and TrkB activity in hippocampal neurons. The activation of TrkA receptors by PACAP required at least 1 h of treatment and did not involve binding to nerve growth factor. Moreover, PACAP induced an increase in activated Akt through a Trk-dependent mechanism that resulted in increased cell survival after trophic factor withdrawal. The increases in Trk and Akt were blocked by K252a, an inhibitor of Trk receptor activity. In addition, transactivation of TrkA receptors by PACAP could be inhibited with PP1, an inhibitor of Src family kinases or BAPTA/AM, (1,2-bis(2-aminophenoxy)ethane-N,N,N,N-tetraacetic acid acetoxymethyl ester), an intracellular calcium chelator. Therefore, PACAP can exert trophic effects through a mechanism involving Trk receptors and utilization of tyrosine kinase signaling. This ability may explain several neuroprotective actions of PACAP upon neuronal populations after injury, nerve lesion, or neurotrophin deprivation.

Cytokine Growth Factor Rev 2002 Feb;13(1):11-17

Distinctive features of Trk neurotrophin receptor transactivation by G protein-coupled receptors.

Lee FS, Rajagopal R, Chao MV.

Department of Psychiatry, Weill Medical College of Cornell University, New York, NY 10021, USA.

Ligands for G protein-coupled receptors (GPCR) are capable of activating mitogenic receptor tyrosine kinases, in addition to the mitogen-activated protein (MAP) kinase signaling pathway and classic G protein-dependent signaling pathways involving adenylyl cyclase and phospholipase. For example, receptors for epidermal growth factor (EGF), insulin-like growth-1 and platelet-derived growth factor and can be transactivated through G protein-coupled receptors. Neurotrophins, such as NGF, BDNF and NT-3 also utilize receptor tyrosine kinases, namely TrkA, TrkB and TrkC. Recently, it has been shown that activation of Trk receptor tyrosine kinases can also occur via a G protein-coupled receptor mechanism, without involvement of neurotrophins. Adenosine and adenosine agonists can activate Trk receptor phosphorylation specifically through the seven transmembrane spanning adenosine 2A (A2A) receptor. Several features of Trk receptor transactivation are noteworthy and differ significantly from other transactivation events. Trk receptor transactivation is slower and results in a selective increase in activated Akt. Unlike the biological actions of other tyrosine kinase receptors, increased Trk receptor activity by adenosine resulted in increased cell survival. This article will discuss potential mechanisms by which adenosine can activate trophic responses

Transactivation of the fibroblast growth factor receptor I (FGFR1)

J Pharmacol Exp Ther 2002 Dec;303(3):909-18

The fibroblast growth factor receptor is at the site of convergence between mu-opioid receptor and growth factor signaling pathways in rat C6 glioma cells.

Belcheva MM, Haas PD, Tan Y, Heaton VM, Coscia CJ.
E. A. Doisy Department of Biochemistry and Molecular Biology, St. Louis University School of Medicine, St. Louis, Missouri 63104, USA.

Mitogenic signaling of G protein-coupled receptors (GPCRs) can proceed via sequential epidermal growth factor receptor (EGFR) transactivation and extracellular signal-regulated kinase (ERK) phosphorylation. Although the mu-opioid receptor (MOR) mediates stimulation of ERK via EGFR transactivation in human embryonic kidney 293 cells, the mechanism of acute MOR signaling to ERK has not been characterized in rat C6 glioma cells that seem to contain little EGFR. Herein, we describe experiments that implicate fibroblast growth factor (FGF) receptor (FGFR) transactivation in the convergence of MOR and growth factor signaling pathways in C6 cells. MOR agonists, endomorphin-1 and morphine, induced a rapid (3-min) increase of ERK phosphorylation that was abolished by MOR antagonist D-Phe-Cys-Tyr-D-Trp-Arg-Thr-Pen-Thr-NH2. By using selective inhibitors and overexpression of dominant negative mutants, data were obtained to suggest that MOR signaling to ERK is transduced by Gbetagamma and entails Ca2+- and protein kinase C-mediated steps, whereas the FGFR branch of the pathway is Ras-dependent. An intermediary role of FGFR1 transactivation was suggested by MOR- but not kappa-opioid receptor (KOR)-induced FGFR1 tyrosine phosphorylation. A dominant negative mutant of FGFR1 attenuated MOR- but not KOR-induced ERK phosphorylation. Thus, a novel transactivation mechanism entailing secreted endogenous FGF may link the GPCR and growth factor pathways involved in MOR activation of ERK in C6 cells.

J Neurochem 2002 May;81(3):506-24

Integrative nuclear FGFR1 signaling (INFS) pathway mediates activation of the tyrosine hydroxylase gene by angiotensin II, depolarization and protein kinase C.

Peng H, Myers J, Fang X, Stachowiak EK, Maher PA, Martins GG, Popescu G, Berezney R, Stachowiak MK.
Department of Pathology and Anatomical Sciences, Molecular and Structural Neurobiology and Gene Therapy Program, State University of New York, Buffalo, New York 14214-3000, USA.

The integrative nuclear FGFR1 signaling (INFS) pathway functions in association with cellular growth, differentiation, and regulation of gene expression, and is activated by diverse extracellular signals. Here we show that stimulation of angiotensin II (AII) receptors, depolarization, or activation protein kinase C (PKC) or adenylate cyclase all lead to nuclear accumulation of fibroblast growth factor 2 (FGF-2) and FGFR1, association of FGFR1 with splicing factor-rich domains, and activation of the tyrosine hydroxylase (TH) gene promoter in bovine adrenal medullary cells (BAMC). The up-regulation of endogenous TH protein or a transfected TH promoter-luciferase construct by AII, veratridine, or PMA (but not by forskolin) is abolished by transfection with a dominant negative FGFR1TK-mutant which localizes to the nucleus and plasma membrane, but not by extracellularly acting FGFR1 antagonists suramin and inositolhexakisphosphate (IP6). Mechanism of TH gene activation by FGF-2 and FGFR1 was further investigated in BAMC and human TE671 cultures. TH promoter was activated by co-transfected HMW FGF-2 (which is exclusively nuclear) but not by cytoplasmic FGF-1 or extracellular FGFs. Promoter transactivation by HMWFGF-2 was accompanied by an up-regulation of FGFR1 specifically in the cell nucleus and was prevented FGFR1(TK-) but not by IP6 or suramin. The TH promoter was also transactivated by co-transfected wild-type FGFR1, which localizes to both to the nucleus and the plasma membrane, and by an exclusively nuclear, soluble FGFR1(SP-/NLS) mutant with an inserted nuclear localization signal. Activation of the TH promoter by nuclear FGFR1 and FGF-2 was mediated through the cAMP-responsive element

(CRE) and was associated with induction of CREB- and CBP/P-300-containing CRE complexes. We propose a new model for gene regulation in which nuclear FGFR1 acts as a mediator of CRE transactivation by AII, cell depolarization, and PKC.

Transactivation of the IGF-1 receptor

Cancer Res 2001 Apr 15;61(8):3294-8

Neutral endopeptidase inhibits neuropeptide-mediated transactivation of the insulin-like growth factor receptor-Akt cell survival pathway.

Sumitomo M, Milowsky MI, Shen R, Navarro D, Dai J, Asano T, Hayakawa M, Nanus DM.
Urologic Oncology Research Laboratory, Department of Urology, Weill Medical College of Cornell University, New York, NY 10021, USA.

G-protein coupled receptor (GPCR) agonists such as neuropeptides activate the insulin-like growth factor-1 receptor (IGF-IR) or the serine-threonine protein kinase Akt, suggesting that neuropeptides-GPCR signaling can cross-communicate with IGF-IR-Akt signaling pathways. Neutral endopeptidase 24.11 (NEP) is a cell-surface peptidase that cleaves and inactivates the neuropeptides endothelin-1 (ET-1) and bombesin, which are implicated in progression to androgen-independent prostate cancer (PC). We investigated the mechanisms of NEP regulation of neuropeptide-mediated cell survival in PC cells, including whether neuropeptide substrates of NEP induce phosphorylations of IGF-IR and Akt in PC cells. Western analyses revealed ET-1 and bombesin treatment induced phosphorylation of IGF-IRbeta and Akt independent of IGF-I in TSU-Pr1, DU145, and PC-3 PC cells, which lack NEP expression, but not in NEP-expressing LNCaP cells. Recombinant NEP and induced NEP expression in TSU-Pr1 cells using a tetracycline-repressive expression system inhibited ET-1-mediated phosphorylation of IGF-IRbeta and Akt, and blocked the protective effects of ET-1 against apoptosis induced by serum starvation. Incubation of TSU-Pr1 cells with specific kinase inhibitors together with ET-1 or bombesin showed that IGF-IR activation is required for neuropeptide-induced Akt phosphorylation, and that neuropeptide-induced Akt activation is predominantly mediated by Src and phosphatidylinositol 3-kinase but not by mitogen-activated protein kinase or protein kinase C. These data show that the neuropeptides ET-1 and bombesin stimulate ligand-independent activation of the IGF-IR, which results in Akt activation, and that this cross-communication between GPCR and IGF-IR signaling is inhibited by NEP.

Relevant references relating to the point that the process of the present invention cannot only be used for inhibitors which inhibit the cleavage catalyzed by metalloproteinase but also for other inhibitors.

Inhibitors of:

Heterotriminar G-proteins:	1.	pertussis toxin
EGF-similiar ligands:	2.	diphtheria toxin or DT mutant CRM197 (for HB-EGF)
	3.	heparin (against heparin-binding growth factors)
	4.	antibodies which are ligand specific and inhibiting
	5.	antisense strategy
Receptor tyrosine kinases:	6.	EGFR inhibitors (in case of EGFR transactivation)

	11.	blocking antibodies of the mentioned receptors
Cytoplasmatic kinases	12.	Src inhibitors
G-protein coupled receptors:	13.	

To the points 1, 4, 6 and 11:

J Immunol 2002 Aug 15; 169(4):2103-10

Complement c3a and c5a induce different signal transduction cascades in endothelial cells.

Schraufstatter IU, Trieu K, Sikora L, Sriramarao P, DiScipio R.

Department of Cancer Biology, La Jolla Institute for Molecular Medicine, San Diego, CA 92121, USA. ingrid@lijimm.org

In leukocytes, C3a and **C5a cause chemotaxis in a G(i)-dependent, pertussis toxin (PT)-**sensitive fashion. Because we found that HUVECs and immortalized human dermal microvascular endothelial cells express small numbers of C3aRs and C5aRs, we asked what the function of these receptors was on these cells. Activation of the C3aR caused transient formation of actin stress fibers, which was not PT-sensitive, but depended on rho activation implying coupling to G(alpha12) or G(alpha13). Activation of the C5aR caused a delayed and sustained cytoskeletal response, which was blocked by PT, and resulted in cell retraction, increased paracellular permeability, and facilitated eosinophil transmigration. C5a, but not C3a, was chemotactic for human immortalized dermal microvascular endothelial cells. **The response to C5a was blocked by inhibitors of** phosphatidylinositol-3-kinase, src kinase, and of **the epidermal growth factor (EGF) receptor (EGFR) as well as by neutralizing Abs against the EGFR and heparin-binding EGF-like factor.** Furthermore, immune precipitations showed that the EGFR was phosphorylated following stimulation with C5a. The C5aR in endothelial cells thus uses a signaling cascade-transactivation of the EGFR-that does not exist in leukocytes, while the C3aR couples to a different G protein, presumably G(alpha12/13).

Zu Punkt 1,7

Mol Pharmacol 2001 Jul;60(1):92-103

Dopamine D(4) and D(2L) Receptor Stimulation of the Mitogen-Activated Protein Kinase Pathway Is Dependent on trans-Activation of the Platelet-Derived Growth Factor Receptor.

Oak JN, Lavine N, Van Tol HH
Laboratory of Molecular Neurobiology, Centre for Addiction and Mental Health, Toronto, Ontario, Canada.

The ability of dopamine D(4) and D(2) receptors to activate extracellular signal-regulated kinases (ERKs) 1 and 2 was compared using Chinese hamster ovary (CHO-K1) cells transfected with D(4.2), D(4.4), D(4.7), and D(2L) receptors. Dopamine stimulation of D(4) or D(2L) receptors produced a transient, dose-dependent increase in ERK1/2 activity. Receptor-specific activation of the ERK mitogen-activated protein kinase (MAPK) pathway was confirmed using the D(2)-like receptor-selective agonist quinpirole, whereas the specific antagonist haloperidol blocked activation. MAPK stimulation was dependent on a **pertussis-toxin-sensitive G protein (G(i/o)).** trans-Activation of the platelet-derived growth factor (PDGF) receptor was an essential step in D(4)

and D(2L) receptor-induced MAPK activation. **PDGF receptor-selective tyrosine kinase inhibitors tyrphostin A9 and AG1295 abolished or significantly inhibited ERK1/2 activation by D(4) and D(2L) receptors.** Dopamine stimulation of the D(4) receptor also produced a rapid increase in tyrosine phosphorylation of the PDGF receptor-beta. The Src-family tyrosine kinase inhibitor PP2 blocked MAPK activation by dopamine; however, this drug was also found to inhibit PDGF-BB-stimulated ERK activity and autophosphorylation of the PDGF receptor-beta. Downstream signaling pathways support the involvement of a receptor tyrosine kinase. The phosphoinositide 3-kinase inhibitors wortmannin and LY294002, protein kinase C inhibitors GF109203X and Calphostin C, dominant-negative RasN17, and the MEK inhibitor PD98059 significantly attenuated or abolished activation of MAPK by dopamine D(4) and D(2L) receptors. Our results indicate that D(4) and D(2L) receptors activate the ERK kinase cascade by first mobilizing signaling by the PDGF receptor, followed by the subsequent activation of ERK1/2 by pathways associated with this receptor tyrosine kinase.

Zu Punkt 2,4,6

Estrogen-induced activation of Erk-1 and Erk-2 requires the G protein-coupled receptor homolog, GPR30, and occurs via trans-activation of the epidermal growth factor receptor through release of HB-EGF.

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Estrogen rapidly activates the mitogen-activated protein kinases, Erk-1 and Erk-2, via an as yet unknown mechanism. Here, evidence is provided that estrogen-induced Erk-1/-2 activation occurs independently of known estrogen receptors, but requires the expression of the G protein-coupled receptor homolog, GPR30. We show that 17beta-estradiol activates Erk-1/-2 not only in MCF-7 cells, which express both estrogen receptor alpha (ER alpha) and ER beta, but also in SKBR3 breast cancer cells, which fail to express either receptor. Immunoblot analysis using GPR30 peptide antibodies showed that this estrogen response was associated with the presence of GPR30 protein in these cells. MDA-MB-231 breast cancer cells (ER alpha-, ER beta+) are GPR30 deficient and insensitive to Erk-1/-2 activation by 17beta-estradiol. Transfection of MDA-MB-231 cells with a GPR30 complementary DNA resulted in overexpression of GPR30 protein and conversion to an estrogen-responsive phenotype. In addition, GPR30-dependent Erk-1/-2 activation was triggered by ER antagonists, including ICI 182,780, yet not by 17alpha-estradiol or progesterone. Consistent with acting through a G protein-coupled receptor, estradiol signaling to Erk-1/-2 occurred via a Gbetagamma-dependent, pertussis toxin-sensitive pathway that required Src-related tyrosine kinase activity and tyrosine phosphorylation of tyrosine 317 of the Shc adapter protein. Reinforcing this idea, estradiol signaling to Erk-1/-2 was dependent upon trans-activation of the epidermal growth factor (EGF) receptor via release of heparan-bound EGF (HB-EGF). **Estradiol signaling to Erk-1/-2 could be blocked by: 1) inhibiting EGF-receptor tyrosine kinase activity, 2) neutralizing HB-EGF with antibodies, or 3) down-modulating HB-EGF from the cell surface with the diphtheria toxin mutant, CRM-197.** Our data imply that ER-negative breast tumors that continue to express GPR30 may use estrogen to drive growth factor-dependent cellular responses

Zu Punkt 2,3,4,6,11

Circ Res 2000 Jul 21;87(2):92-8

Heparin blockade of thrombin-induced smooth muscle cell migration involves inhibition of epidermal growth factor (EGF) receptor transactivation by heparin-binding EGF-like growth factor.

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Agonists of G protein-coupled receptors, such as thrombin, act in part by transactivating the epidermal growth factor (EGF) receptor (EGFR). Although at first a ligand-independent mechanism for EGFR transactivation was postulated, it has recently been shown that this transactivation by various G protein-coupled receptor agonists can involve heparin-binding EGF-like growth factor (HB-EGF). Because thrombin stimulation of vascular smooth muscle cell migration is blocked by heparin and because heparin can displace HB-EGF, we investigated the possibility that thrombin stimulation of smooth muscle cells (SMCs) depends on EGFR activation by HB-EGF. In rat SMCs, **EGFR phosphorylation and extracellular signal-regulated kinase (ERK) activation in response to thrombin are inhibited not only by the EGFR inhibitor AG1478 and by EGFR blocking antibody but also by heparin and by neutralizing HB-EGF antibody**. HB-EGF-dependent signaling induced by thrombin is inhibited by batimastat, which suggests a requirement for pro-HB-EGF shedding by a metalloproteinase. We further demonstrate that this novel pathway is required for the migration of rat and baboon SMCs in response to thrombin. We conclude from these data that the inhibitory effect of heparin on SMC migration induced by thrombin relies, at least in part, on a blockade of HB-EGF-mediated EGFR transactivation.

Zu Punkt 5
J Cell Biol 2002 Jul 22;158(2):221-6

The metalloprotease Kuzbanian (ADAM10) mediates the transactivation of EGF receptor by G protein-coupled receptors.

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Communication between different signaling pathways enables cells to coordinate the responses to diverse environmental signals. Activation of the transmembrane growth factor precursors plays a critical role in this communication and often involves metalloprotease-mediated proteolysis. Stimulation of G protein-coupled receptors (GPCR) transactivates the EGF receptors (EGFRs), which occurs via a metalloprotease-dependent cleavage of heparin-binding EGF (HB-EGF). However, the metalloprotease mediating the transactivation remains elusive. We show that the integral membrane metalloprotease Kuzbanian (KUZ; ADAM10), which controls Notch signaling in Drosophila, stimulates GPCR transactivation of EGFR. Upon stimulation of the bombesin receptors, KUZ increases the docking and activation of adaptors Src homology 2 domain-containing protein and Gab1 on the EGFR, and activation of Ras and Erk. In contrast, transfection of a protease domain-deleted KUZ, or **blocking endogenous KUZ by morpholino antisense oligonucleotides, suppresses the transactivation**. The effect of KUZ on shedding of HB-EGF and consequent transactivation of the EGFR depends on its metalloprotease activity. GPCR activation

enhances the association of KUZ and its substrate HB-EGF with tetraspanin CD9. Thus, KUZ regulates the relay between the GPCR and EGFR signaling pathways.

Zu Punkt 4 (TGF alpha blockierender Antikörper)

J Biol Chem 2002 Nov 8;277(45):42603-12

Transactivation of the epidermal growth factor receptor in colonic epithelial cells by carbachol requires extracellular release of transforming growth factor-alpha.

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We have shown previously that the muscarinic agonist, carbachol (CCh), transactivates the epidermal growth factor receptor (EGFr) via calmodulin, Pyk-2, and Src kinase activation. EGFr phosphorylation causes extracellular signal-regulated kinase (ERK) activation and inhibits CCh-stimulated chloride secretion across intestinal epithelial cells. Here we investigated whether CCh-stimulated EGFr transactivation involves EGFr ligand release. Pre-incubation of T(84) cell monolayers with a neutralizing antibody to the EGFr ligand binding domain decreased CCh-induced phosphorylation of EGFr and ERK. CCh-stimulated efflux of (86)Rb+ from T(84) cell monolayers, which parallels changes in chloride secretion, was potentiated by anti-EGFr pre-incubation. Anti-EGFr did not reduce CCh-stimulated Pyk-2 phosphorylation. Co-incubation with the Src kinase inhibitor PP2 and anti-EGFr had an additive inhibitory effect on CCh-induced ERK phosphorylation greater than either inhibitor alone. CCh caused the basolateral release of transforming growth factor alpha (TGF-alpha) into T(84) cell bathing media. A metalloproteinase inhibitor, WAY171318, reduced CCh-induced phosphorylation of ERK and completely blocked EGFr phosphorylation and TGF-alpha release. We conclude that CCh-stimulated EGFr transactivation and subsequent ERK activation, a pathway that limits CCh-induced chloride secretion, is mediated by metalloproteinase-dependent extracellular release of TGF-alpha and intracellular Src activation. These findings have important implications for our understanding of the role of growth factors in regulating epithelial ion secretion.

Zu Punkt 4,6,12

Nat Med 2002 Mar;8(3):289-93

Prostaglandin E2 transactivates EGF receptor: a novel mechanism for promoting colon cancer growth and gastrointestinal hypertrophy.

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Prostaglandins (PGs), bioactive lipid molecules produced by cyclooxygenase enzymes (COX-1 and COX-2), have diverse biological activities, including growth-promoting actions on gastrointestinal mucosa. They are also implicated in the growth of colonic polyps and cancers. However, the precise mechanisms of these trophic actions of PGs remain unclear. As activation of the epidermal growth factor receptor (EGFR) triggers mitogenic signaling in gastrointestinal mucosa, and its expression is also upregulated in colonic cancers and most neoplasms, we investigated whether PGs transactivate EGFR. Here we provide evidence that prostaglandin E2 (PGE2) rapidly phosphorylates EGFR and triggers the extracellular signal-regulated kinase 2 (ERK2)--mitogenic signaling pathway in normal gastric epithelial (RGM1) and colon cancer (Caco-2, LoVo and

HT-29) cell lines. **Inactivation of EGFR kinase with selective inhibitors** significantly reduces PGE2-induced ERK2 activation, c-fos mRNA expression and cell proliferation. **Inhibition of matrix metalloproteinases (MMPs), transforming growth factor-alpha (TGF-alpha) or c-Src blocked PGE2-mediated EGFR transactivation** and downstream signaling indicating that PGE2-induced EGFR transactivation involves signaling transduced via TGF-alpha, an EGFR ligand, likely released by c-Src-activated MMP(s). Our findings that PGE2 transactivates EGFR reveal a previously unknown mechanism by which PGE2 mediates trophic actions resulting in gastric and intestinal hypertrophy as well as growth of colonic polyps and cancers.

To point 13

Prostate 2000 Jul 1;44(2):172-80

Potentiation of the inhibitory effect of growth hormone-releasing hormone antagonists on PC-3 human prostate cancer by bombesin antagonists indicative of interference with both IGF and EGF pathways.

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BACKGROUND: In view of the involvement of various neuropeptides and growth factors in the progression of androgen-independent prostate cancer, we investigated the effects of antagonists of growth hormone-releasing hormone (GHRH) alone or in combination with an antagonist of bombesin/gastrin-releasing peptide (BN/GRP) on PC-3 human prostate cancers. METHODS: Nude mice implanted with PC-3 tumors received GHRH antagonists MZ-5-156 or JV-1-38, each at 20 microgram/day s.c. In experiment 2, treatment consisted of daily injections of JV-1-38 (20 microgram), BN/GRP antagonist RC-3940-II (10 microgram), or a combination of JV-1-38 and RC-3940-II. Serum IGF-I levels, expression of mRNA for IGF-II, and characteristics of BN/GRP and EGF receptors in tumor tissue were investigated. RESULTS: JV-1-38 induced a greater inhibition of tumor growth and suppression of IGF-II mRNA than MZ-5-156, both compounds causing a similar decrease in serum IGF-I. In experiment 2, JV-1-38 and RC-3940-II produced a comparable reduction in tumor volume (65% and 61%, respectively), but a combination of both antagonists augmented tumor inhibition to 75%. Combined treatment with JV-1-38 and RC-3940-II also led to a greater suppression of IGF-II mRNA (92%), as compared with JV-1-38 (72%) or RC-3940-II (77%). Serum IGF-I concentration was lowered only in mice treated with JV-1-38, while the downregulation of BN/GRP and EGF receptors was specific for groups receiving RC-3940-II. CONCLUSIONS: The inhibitory effects of GHRH antagonists on PC-3 human androgen-independent prostate cancer can be potentiated by concomitant use of BN/GRP antagonists. The combination of both types of analogs apparently interferes with both IGF and bombesin/EGF pathways, and might be clinically useful for the management of androgen-independent prostate cancer.

Transaktivierung in normalen, nicht transformierten Zellen:

Unsere Ausgangspublikation zeigt die EGFR Transaktivierung in HEK293 und COS-7 Zellen (beides keine Krebszelllinien)

Nature 1999 Dec 23-30;402(6764):884-8

EGF receptor transactivation by G-protein-coupled receptors requires metalloproteinase cleavage of proHB-EGF.

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Cross-communication between different signalling systems allows the integration of the great diversity of stimuli that a cell receives under varying physiological situations. The transactivation of epidermal growth factor receptor (EGFR)-dependent signalling pathways upon stimulation of G-protein-coupled receptors (GPCRs), which are critical for the mitogenic activity of ligands such as lysophosphatidic acid, endothelin, thrombin, bombesin and carbachol, provides evidence for such an interconnected communication network. Here we show that EGFR transactivation upon GPCR stimulation involves proHB-EGF and a metalloproteinase activity that is rapidly induced upon GPCR-ligand interaction. We show that inhibition of proHB-EGF processing blocks GPCR-induced EGFR transactivation and downstream signals. The pathophysiological significance of this mechanism is demonstrated by inhibition of constitutive EGFR activity upon treatment of PC3 prostate carcinoma cells with the metalloproteinase inhibitor batimastat. Together, our results establish a new mechanistic concept for cross-communication among different signalling systems.

Prostate 2003 Jan 1;54(1):1-7

Transactivation of ErbB1 and ErbB2 receptors by angiotensin II in normal human prostate stromal cells.

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BACKGROUND: Heparin-binding epidermal growth factor-like growth factor (HB-EGF) is synthesized primarily in the stromal compartment of the human prostate and may regulate stromal as well as epithelial cell growth and survival. The primary cognate HB-EGF receptor, ErbB1, has been shown recently to be transactivated by G-protein coupled receptors (GPCRs) through regulated proteolytic cleavage of the membrane-bound, precursor form of HB-EGF. Previous studies have demonstrated that human prostate tissue, especially tissue from benign prostatic hyperplasia (BPH), has high angiotensin converting enzyme activity, and a high density of angiotensin (Ang) receptors in periurethral stromal cells. Because the pressor peptide Ang II signals through GPCRs, we tested the possibility that Ang II could transactivate ErbB1/ErbB2 in human prostate stromal (hPS) cells. METHODS: Primary and early passage hPS cells were used as an in vitro model. Cells were stimulated by recombinant HB-EGF or Ang II and total cell lysates were harvested for immunoprecipitation and Western blot. Cell growth was measured by [(3)H]thymidine incorporation assay and 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazoliumbromide (MTT) assay. RESULTS: Ang II receptors AT1 and AT2 were expressed in hPS cells. ErbB1 and ErbB2 receptors were activated by HB-EGF. Furthermore, Ang II was able to transactivate both ErbB1 and ErbB2, and this transactivation activity could be abolished by pretreatment with [Glu-52]-diphtheria toxin/CRM197, a specific inhibitor of HB-EGF bioactivity. Consistent with its transactivation activity, Ang II modestly promoted hPS cell growth and this effect was abolished by pretreatment with the ErbB1 antagonist AG1478. CONCLUSION: These experiments suggest a regulatory role for Ang II in the prostate stroma and implicate the endogenous stromal growth factor HB-EGF as a mediator of Ang II signaling in the prostate.

J Biol Chem 2002 Dec 13;277(50):48755-63

Gastrin stimulates cyclooxygenase-2 expression in intestinal epithelial cells through multiple signaling pathways. Evidence for involvement of ERK5 kinase and transactivation of the epidermal growth factor receptor.

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Gastrin is a hormone produced by G-cells in the normal gastric antrum. However, colorectal carcinoma cells may aberrantly produce gastrin and exhibit increased expression of cholecystokinin B (CCK-B)/gastrin receptors. Gastrin is trophic for the normal gastric oxyntic mucosa and exerts a growth-promoting action on gastrointestinal malignancy. Thus, gastrin may act as an autocrine/paracrine or endocrine factor in the initiation and progression of colorectal carcinoma. The molecular mechanisms involved have not been elucidated. Hypergastrinemia induced by Helicobacter pylori infection is associated with increased cyclooxygenase-2 (COX-2) expression in gastric and colorectal tissues, suggesting the possibility that gastrin up-regulates COX-2 expression in these tissues; this has not been confirmed. We report here that gastrin significantly increases the expression of COX-2 mRNA and protein, the activity of the COX-2 promoter, and the release of prostaglandin E(2) from a rat intestinal epithelial cell line transfected with the CCK-B receptor. These actions were dependent upon the activation of multiple MAPK signal pathways, including ERK5 kinase; transactivation of the epidermal growth factor receptor; and the increased expression and activities of transcription factors ELK-1, activating transcription factor-2, c-Fos, c-Jun, activator protein-1, and myocyte enhancer factor-2. Thus, our findings identify the signaling pathways coupling the CCK-B receptor with up-regulation of COX-2 expression. This effect may contribute to this hormone-dependent gastrointestinal carcinogenesis, especially in the colon.

Biochem J 2003 Jan 21;Pt [epub ahead of print]

Epidermal growth factor receptor and metalloproteinases mediate thromboxane A 2- dependent extracellular signal-regulated kinase activation.

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The signalling pathways linking G-protein-coupled receptors to mitogen-activated protein kinases involve receptor and non-receptor tyrosine kinases and protein kinase C (PKC). We explored the pathways implicated in the thromboxane (TX) A 2- dependent activation of extracellular signal-regulated protein kinase (ERK) and the role of the two TX receptor (TP) isoforms, TPalpha and TPbeta. ERK activation by IBOP, a TX analogue, was dependent on epidermal growth factor (EGF) receptor in TPalpha or TPbeta transfected cells and in human aortic smooth muscle cells (hASMC) since AG1478, a selective inhibitor of tyrosine phosphorylation of EGF receptor, strongly blocked ERK and EGF receptor phosphorylation. In addition, transactivation leading to ERK activation involved matrix metalloproteinases (MMP) since BB2516, an inhibitor of MMP, reduced ERK and EGFR phosphorylation in TPalpha or TPbeta transfected cells. Moreover, we showed that both isoforms activate ERK phosphorylation in a Src kinase-dependent manner whereas PKC was mainly implicated in ERK activation and EGFR phosphorylation by TPbeta. In hASMC, we showed that ERK activation depended on both pertussis sensitive and insensitive

Galpha proteins. We further demonstrated that EGF receptor, PKC, Src kinase and MMP are involved in ERK activation by TX. Our results highlight a role of MMP and PKC in the transactivation triggered by the thromboxane receptors and demonstrate this mechanism in primary cells, i.e. hASMC

Int J Mol Med 2001 Mar;7(3):329-34

Transmodulation of epidermal growth factor receptor mediates IL-1 beta-induced MMP-1 expression in cultured human keratinocytes.

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Ultraviolet (UV) irradiation causes human skin aging and skin cancer through the activation of matrix metalloproteinases (MMPs) which are responsible for the degradation of collagen and tumor progression in human skin. The molecular mechanisms of UV-induced MMPs are yet to be defined. Our previous studies and others suggest that i) the transient activation of cell surface receptors and subsequent activation of MAP kinase cascade contributes to the transcriptional up-regulation of MMPs; and ii) UV-induced expression of pro-inflammatory cytokines such as IL-1 beta and TNF-alpha may also account for the expression of MMPs. However, signaling pathway through which cytokines induce MMP expression remains to be unraveled. In this study, we investigated the pathway that leads to the IL-1 beta-induced up-regulation of MMP-1 in human keratinocytes. IL-1 beta activated epidermal growth factor (EGF) receptor in cultured human keratinocytes in a time- and dose-dependent manner. IL-1 beta-induced EGF receptor tyrosine phosphorylation started at 5 min and peaked at 10 min and remained elevated up to 40 min post IL-1 beta treatment. EGF receptor kinase inhibitor PD153035 and AG1478 inhibited IL-1 beta-induced EGF receptor tyrosine phosphorylation. To test the effect of EGF receptor transactivation on downstream components, we examined the ERK activation by IL-1 beta. We found that IL-1 beta-induced ERK phosphorylation, PD153035 and MEK inhibitor PD98059 blocked IL-1 beta-induced ERK activity. Furthermore, both inhibitors also dramatically reduced IL-1 beta-induced expression of c-jun and c-fos mRNA which are required for up-regulation of MMPs. EGF receptor kinase inhibitor PD153035 and AG1478 and MEK inhibitor PD98059 also blocked IL-1 beta induction of MMP-1 in cultured human keratinocytes. Collectively, our data indicate that IL-1 beta-induced expression of MMP-1 is mediated by transactivation of EGF receptor and through ERK pathway in human keratinocytes.

Nat Med 2002 Jan;8(1):35-40

Cardiac hypertrophy is inhibited by antagonism of ADAM12 processing of HB-EGF: metalloproteinase inhibitors as a new therapy.

Asakura M, Kitakaze M, Takashima S, Liao Y, Ishikura F, Yoshinaka T, Ohmoto H, Node K, Yoshino K, Ishiguro H, Asanuma H, Sanada S, Matsumura Y, Takeda H, Beppu S, Tada M, Hori M, Higashiyama S.

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G-protein-coupled receptor (GPCR) agonists are well-known inducers of cardiac hypertrophy. We found that the shedding of heparin-binding epidermal growth factor (HB-EGF) resulting from metalloproteinase activation and subsequent transactivation of the epidermal growth factor receptor

occurred when cardiomyocytes were stimulated by GPCR agonists, leading to cardiac hypertrophy. A new inhibitor of HB-EGF shedding, KB-R7785, blocked this signaling. We cloned a disintegrin and metalloprotease 12 (ADAM12) as a specific enzyme to shed HB-EGF in the heart and found that dominant-negative expression of ADAM12 abrogated this signaling. KB-R7785 bound directly to ADAM12, suggesting that inhibition of ADAM12 blocked the shedding of HB-EGF. In mice with cardiac hypertrophy, KB-R7785 inhibited the shedding of HB-EGF and attenuated hypertrophic changes. These data suggest that shedding of HB-EGF by ADAM12 plays an important role in cardiac hypertrophy, and that inhibition of HB-EGF shedding could be a potent therapeutic strategy for cardiac hypertrophy.

Biochem Biophys Res Commun 2002 Sep 20;297(2):375-81

A role of heparin-binding epidermal growth factor-like growth factor in cardiac remodeling after myocardial infarction.

Heparin-binding epidermal growth factor-like growth factor (HB-EGF) is known to induce cell growth in various cell types via transactivation of epidermal growth factor receptor (EGFR). To investigate the involvement of HB-EGF and EGFR in cardiac remodeling after myocardial infarction (MI), we examined the expressions of mRNA and protein in rat hearts 6 weeks after MI-induction. Where increased expressions of HB-EGF mRNA and protein were observed, infarcted myocardium was replaced by extracellular matrix and interstitial fibroblasts. EGFR mRNA and protein expression did not show significant changes in sham-operated heart tissues, non-infarcted region, and infarcted region. In vitro study demonstrated that HB-EGF mRNA was expressed mainly in cultured fibroblasts rather than in myocytes. We suggest that the interaction between HB-EGF and EGFR transactivation is closely related to the proliferation of cardiac fibroblasts and cardiac remodeling after MI in an autocrine, paracrine, and juxtacrine manner.

Diabetes 2003 Jan;52(1):124-32

Glucagon-like peptide 1 induces pancreatic beta-cell proliferation via transactivation of the epidermal growth factor receptor.

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We previously provided evidence that glucagon-like peptide 1 (GLP-1) induces pancreatic beta-cell growth nonadditively with glucose in a phosphatidylinositol (PI) 3-kinase- and protein kinase C zeta-dependent manner. However, the exact mechanism by which the GLP-1 receptor (GLP-1R), a member of the G protein-coupled receptor (GPCR) superfamily, activates the PI 3-kinase signaling pathway to promote beta-cell growth remains unknown. We hypothesized that the GLP-1R could activate PI 3-kinase and promote beta-cell proliferation through transactivation of the epidermal growth factor (EGF) receptor (EGFR), an event possibly linked to GPCRs via activation of c-Src and the production of putative endogenous EGF-like ligands. Both the c-Src inhibitor PP1 and the EGFR-specific inhibitor AG1478 blocked GLP-1-induced [(3)H]thymidine incorporation in INS(832/13) cells as well as in isolated rat islets, while only AG1478 inhibited the proliferative action of betacellulin (BTC), an EGFR agonist. Both compounds also suppressed GLP-1-induced PI 3-kinase activation. A time-dependent increase in tyrosine phosphorylation of the EGFR in response to GLP-1 was observed in INS(832/13) cells. This transactivation of the EGFR was sensitive to both the pharmacological agents PP1 and AG1478. The action of GLP-1 and BTC on INS cell proliferation was found to be not additive. Overexpression of a dominant-negative EGFR

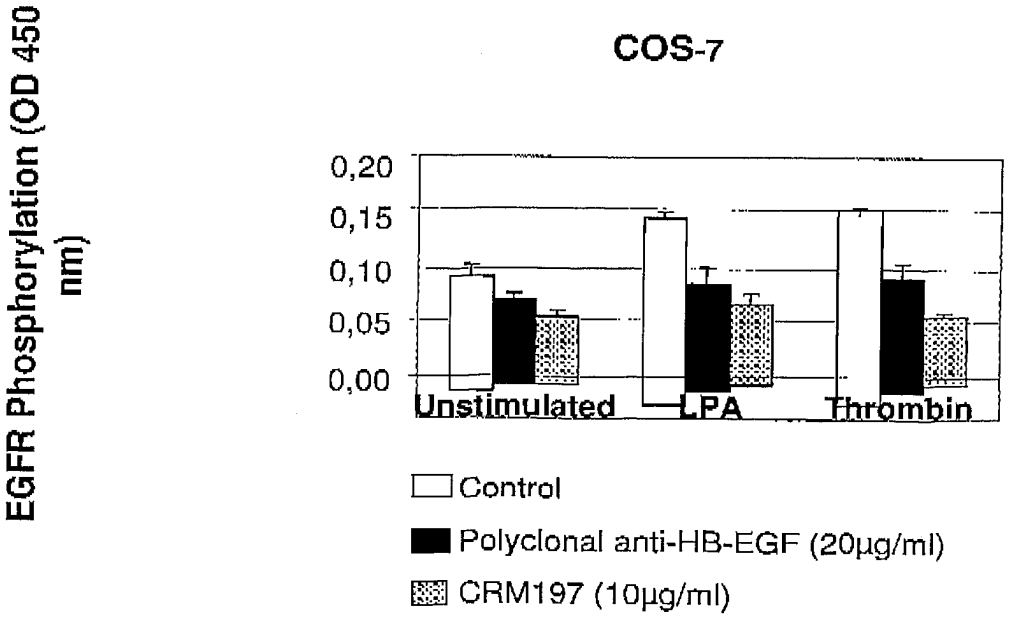
in INS cells with a retroviral expression vector curtailed GLP-1-induced beta-cell proliferation. GLP-1 treatment of INS cells caused a decrease in cell surface-associated BTC, as shown by FACS analysis. Also, the metalloproteinase inhibitor GM6001 and an anti-BTC neutralizing antibody suppressed the GLP-1 proliferative effect. Finally, coculturing the prostatic cancer cell line LNCaP that lacks GLP-1 responsiveness with INS cells increased LNCaP cell proliferation in the presence of GLP-1, thus revealing that INS cells secrete a growth factor in response to GLP-1. GM6001 and an anti-BTC neutralizing antibody suppressed increased LNCaP cell proliferation in the presence of GLP-1 in the coculture experiments. The results are consistent with a model in which GLP-1 increases PI 3-kinase activity and enhances beta-cell proliferation via transactivation of the EGFR that would require the proteolytic processing of membrane-anchored BTC or other EGF-like ligands.

In order to identify additional inhibitors of GPCR-induced EGFR-transactivation, we examined the effect of anti-HB-EGF antibodies on the stimulation of EGFR phosphorylation by the GPCR agonist LPA and Thrombin in COS-7 cells. To this end, appropriately treated cells were lysed and tyrosine phosphorylation of the EGFR analysed by colorimetric enzyme-linked immunosorbent assay (ELISA).

Specifically, COS-7 cells were seeded in 6-well plates and cultured overnight at 37°C in 5% CO_2. Following starvation for 24 h, cells were preincubated for 1 h with polyclonal anti HB-EGF antibodies (20µg/ml) or with the diphtheria toxin mutant CRM197 (10µg/ml). Finally, cells were stimulated in triplicate with the GPCR agonists LPA (10µM) or Thrombin (2U/ml) for 3 min at 37°C and lysed in 400µl lysis buffer (150 mM NaCl, 50 mM Hepes pH 7.5, 10 % Glycerin, 5 mM EDTA pH 8.0, 1 % Triton-X 100, 20 mM sodium pyrophosphate, 10 µg/ml aprotinin, 1 mM PMSF, 2 mM sodium orthovanadate, 100 mM NaF). After incubation on ice for 10 min lysates (85 µl/well) were transferred to an ELISA microtiter plate (Nunc Maxisorb), which had been coated overnight at 4°C with a monoclonal anti-EGFR Ab (mAb 108.1 at 1.0 µg /ml; 100µl/well), blocked with 150 µl/well blocking buffer (PBS, 0.5% BSA) and washed 6 times with washing buffer (PBS, 0.05% Tween 20) using an automated plate washer. Lysates were incubated on the antibody-coated plate o/n at 4 °C with gentle agitation. Following washing (4X) with 100 µl washing buffer, 100 µl of biotinylated anti-phosphotyrosine monoclonal antibody 4G10 (UBI) diluted to 0.2 µg/ml in dilution buffer (PBS, 0.5% BSA, 0.05% Tween 20, 5 mM EDTA) was added to each well and incubated for 2 h at RT. The plate was washed and 100µl HRP-conjugated streptavidin (UBI) diluted 1:40000 in dilution buffer was added to each well and incubated for 30 min at RT. Free avidin conjugate was washed away (3X) and 100µl substrate solution (tetramethyl benzidine, TMB, Calbiochem) was added to each well and incubated

at RT with gently shaking in the dark. After 15 min the reaction was stopped by addition of 100 µl/well 250 mM HCl and the absorbance at 450 nm was read with a reference wavelength of 650 nm using a microplate reader (Thermo Lab Systems).

As depicted below, GPCR agonists LPA and Thrombin induce the tyrosine phosphorylation of the EGFR in COS-7 cells (which is monitored as a change in absorbance at 450 nM). This EGFR transactivation is completely inhibited by treatment with anti HB-EGF antibodies or the diphtheria toxin mutant CRM197. This demonstrates that inhibitors, which neutralize the activity of HB-EGF interfere strongly with GPCR-mediated EGFR activation as has been observed for metalloprotease inhibitors such as batimastat. In addition, this approach provides a rationale for therapeutic antibody treatment of hyperproliferative diseases, which are associated with disturbed GPCR-induced receptor tyrosine kinase signalling.



In the letter dated 29.01.2003 Dr. Weiss writes that the examiner has asked for the claims to be modified to the extend that cells are to be contacted with an agent that initiates the GPCR-mediated signal transduction pathway.

As a matter of fact, activation of the GPCR-mediated signal transduction pathway by such agents represents an endogenous feature of the autocrine growth stimulation of cells that is targeted by the inhibitors disclosed in the present invention.

For example, it is described in the patent application that human PC-3 prostate carcinoma cells have been reported to utilize EGFR-dependent pathways for growth promotion and also produce and are responsive to the GPCR peptide ligand bombesin (ref. 27, 28 of WO 01/12182). As a tool for dissecting and defining the individual components of the underlying signalling mechanism in vitro, it is shown in Fig. 4f of the present application how PC-3 cells were washed extensively and subsequently contacted with specific GPCR-stimulating agents in order to assess their effect on EGFR phosphorylation. However, as described subsequently in Fig. 4g of the patent application, when PC-3 cells are cultured normally, the EGFR is activated constitutively (as demonstrated by its high level of phosphorylation) <u>without</u> addition of any specific GPCR-stimulating agents and the protease inhibitor BB-94 (batimastat) completely inhibits this constitutive EGFR phosphorylation, indicating that the continuous growth stimulation by endogenously produced GPCR ligands is interrupted. This assay can be used for identification of inhibitors of GPCR-mediated receptor tyrosine kinase activation in vitro and is representative of pathophysiological autocrine growth stimulation *in vivo*, e. g. in the case of prostate hyperplasia, whereby the constitutive production and presence of GPCR-

ligands in the blood that is elicited via receptor tyrosine kinase activation in turn causes continuous GPCR stimulation and thus further receptor tyrosine kinase activity.